Heterologous Comparative Genomics to Identify Candidate Genes Impacting Fruit Quality in Apple (*Malus* x *domestica* Borkh.)

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Abstract

Fruits are an important source of healthy compounds in the human diet, such as fibers, organic acids, vitamins, and antioxidants. All these metabolites are developed during fruit ripening, a complex process characterized by dramatic physiological changes. Fruit ripening is normally distinguished as climacteric or non-climacteric, depending on the direct effect of the hormone ethylene in the control of these processes, especially on fruit softening. Variation in fruit firmness, one of the most evident phenomena, strongly influences the general fruit quality, limiting the shelf life due to a minor resistance to post-harvest diseases. To investigate the transcription dynamics over the apple ripening, two wide genomic cDNA microarrays were used: an apple specific homologous system, and a tomato heterologous platform. In this study, we assessed the potential of the heterologous array to analyze the apple ripening physiology. The normal ripening transcriptome was compared with the distorted one obtained with 1-MCP treatment (an ethylene competitor), allowing for the identification of a comprehensive gene set, developmental and ethylene dependent.

INTRODUCTION

Fruits, in general, are an essential component of the human diet providing healthy nutraceutical compounds (Giovannoni, 2004). All the features defining high quality fruits are determined and developed during the ripening process (Lelièvre et al., 1997), a physiological syndrome distinguished in two general behaviors: climacteric or non-climacteric (Alexander and Grierson, 2002). The first category, to which apple and tomato belong, differs from the second for the starch accumulation occurring during the maturation phase, and the characteristic ethylene burst at the climacteric stage. Ethylene is a gaseous hormone able to trigger and coordinate all the physiological changes of fruit ripening (Alexander and Greirson, 2002), controlling in particular the action of several cell wall enzymes, responsible for the loss of firmness. This aspect highly impacts the fruit economic value, limiting fruit shipping and storability, because a fruit with a reduced shelf life becomes more prone to post-harvest diseases and mechanical damage.

To unravel the genetic bases responsible for all these ripening changes, the identification of the gene set putatively involved in these pathways is challenging.

To date, microarray technique is one of the most promising available technologies for candidate gene discovery at a wide genomic scale. This strategy has already been used for studying gene expression related to fruit ripening in strawberry (Aharoni et al., 2002) and peach (Trainotti et al., 2006).

Heterologous system has been described in studies on closely related species belonging to the same botanical family, like Brassicaceae, Leguminoseae, and Graminaceae (Van de Martel and Aarts, 2006). In the Solanaceae family, TOM1 array was used in a comprehensive scale comparison, anchoring to tomato the functional profile of two related species: eggplant and pepper (Moore et al., 2005). Tomato digital expression data was further used in a more genetic distant comparison with the available grape ESTs collection, identifying common genes important for the ripening of these two species (Fei et al., 2004), of which tomato has a climacteric behavior, while grape has a non-climacteric ripening type.

The first cross-family comparative genomic in plants was carried out in dicots, where cDNA arrays of *Arabidopsis* was used to analyze genes involved in different physiological processes of other species, like *Avena fatua*, *Populus deltoids*, and *Euphorbia esula* (Horvath et al., 2003).

In this work, we presented a preliminary translational tomato – apple genomic comparison, addressed to target candidate genes putatively involved in the control of fruit quality, with the final aim to develop functional markers useful in breeding programs.

MATERIALS AND METHODS

Ripening Evolution

Fruit development and ripening of the apple cultivar 'Mondial Gala' was followed over the summer 2005, identifying five main ripening stages: (i) Green, 60 DAFB (days after full bloom); (ii) Breaker, 91 DAFB; (iii) Red Ripe, 114 DAFB; (iv) T1, 120 DAFB, and (v) T2, 123 DAFB. The Red Ripe stage corresponds also to the harvest, which was defined by a starch degradation value of 7 (on a 1 to 10 scale). At harvest a sub-sample of 40 fruits was treated with 1 ppm of 1-MCP, in a sealed and ventilated container for 12 hours. After the treatment the two sub-samples (control/1-MCP) were stored in room temperature condition.

For these five stages, ethylene assessment was carried out with a DANI gas-chromatographer, and firmness was measured with an Effegi penetrometer (11 mm probe). Fruit softening was defined as difference between the firmness value at harvest, and after a post-harvest period.

RNA Isolation and Array Hybridization

Flesh tissue collected from the seven samples (Green, Breaker, Red Ripe, $T1_{Ctrl/1\text{-}MCP}$ and $T2_{Ctrl/1\text{-}MCP}$) were frozen in liquid nitrogen and immediately stored at -80°C. RNA was isolated following the method based on CTAB extraction buffer (Zeng et al., 2002). cDNA synthesis, fluorescent labeling and the slide scanning procedure were performed as reported in Alba et al. (2004) and in the TED web site (http://ted.bti.cornell.edu).

In this work, two cDNA microarrays (Hom & Het) have been used. The homologous array apple specific was realized within the HiDRAS consortium (Soglio et al., 2006) with ~1,600 cDNA spots. The heterologous chip is represented by the TOM1, an array specifically designed for tomato, with ~13,000 cDNA spots (Alba et al., 2004 and http://www.bti.cornell.edu/CGEP/CGEP.html).

Statistical Analysis

Functional hom & het microarray comparison were carried out, adapting a common reference experimental design. For both systems were performed a minimum of three biological replicates/hybridization. Microarray digitalized expression data were analyzed using BRB-Array Tool, software developed by Richard Simon and Amy Lam (http://linus.nci.nih.gov/BRB-ArrayTool.html).

Genetic Mapping

To map the candidate genes identified, we used the 'Fuji' x 'Mondial Gala'

population (Costa et al., unpublished). This progeny consists of 176 seedlings, genotyped with 221 markers. Genetic map was built using JoinMap 3.0 (Van Ooijen and Voorips, 2001).

RESULTS

'Mondial Gala' is an early apple variety, which in 2005 in Northern Italy reached the commercial harvest at 114 dDAFB. Before harvest the amount of ethylene remained at a low pre-climacteric concentration, almost undetectable. At the Red Ripe stage, we analyzed the starting of the climacteric phase, associated with the typical ethylene burst. At 120 DAFB ($T1_{Ctrl}$) the ethylene production was 16 $\mu l\ kg^{-1}\ h^{-1}$, a value that rose up to 52.67 $\mu l\ kg^{-1}\ h^{-1}$ in the $T2_{Ctrl}$ sample (Table 1).

Fruit firmness measured at these two stages reported a value of 7.53 $kg{\cdot}cm^{-2}$ and 6.03 $kg{\cdot}cm^{-2}$ for $T1_{Ctrl}$ and $T2_{Ctrl}$ respectively, identifying a value of 1.5 $kg{\cdot}cm^{-2}$ (Table 1).

1-MCP treatment, applied at harvest caused a relevant physiological distortion in the normal post-harvest ripening, strongly affecting the ethylene production and the fruit softening. In the treated sample the ethylene production never showed the climacteric peak: in $T1_{1\text{-}MCP}$ the ethylene was 0.66 $\mu l\ kg^{-1}\ h^{-1}$ and in $T2_{1\text{-}MCP}$ 0.42 $\mu l\ kg^{-1}\ h^{-1}$. Similarly to the ethylene evolution, significant changes were not observed in the fruit firmness.

The 652 heterologous genes differentially expressed were organized in 24 functional annotated categories, while the 139 homologous genes of apple were sorted in 19 functional classes.

In this experiment we identified genes involved in processes like cell wall polysaccharides degradation, ethylene production/biosynthesis, and transcriptional regulation, which are physiological events related to the fruit softening process.

Two of these genes, *ACO* (1-aminocyclopropane-1-carboxylic acid oxidase - ethylene production) and *PG* (polygalacturonase - cell wall metabolism), were used for genetic mapping studies. Functional markers based on these two candidate genes were mapped on the linkage group 10 of 'Mondial Gala' (Costa et al., 2005).

DISCUSSION

In this study we present a cross-family heterologous functional comparison, hybridizing apple RNA on both tomato and apple array, aiming at the identification of the gene set involved in apple ripening. Comparative genomics became a challenging research in the last few years for the attempt made in the study of the transcription dynamics in "non-model" species, which differs from the "model" plants basically for the lack of genomic resource tools and information availability. However, the discovery of important genes controlling economical important traits could represent a "hot topic" due to the agronomical relevance of some non-model plants.

The heterologous hybridization could provide a cost-effective alternative to the de novo construction of high dense species-specific microarrays, which are normally expensive and laborious.

In literature, examples of successfully heterologous hybridization within and between botanical families have been already presented (Horvath et al., 2003; Renn et al., 2004). In this work, we used tomato as a model species, because of the high dense genomic platforms and the several specific mutants, which have highlighted the impact of some important genes in the ripening process (Moore et al., 2002). The heterologous gene set, together with the homologous differentially expressed genes, allowed us to reveal a good level of consistency between the two genomic platforms.

We extended this information complex from comparative genomics to translational genomics (Stacey and Van den Bosch, 2005), as it was presented in the Leguminoseae initiative. This new genomic concept implies the use of a wide genomic outcome into agricultural applications, using new molecular markers to support genotype selection and association studies for important traits.

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Tables

Table 1. Phenotypic characterization of the seven apple samples used in the functional transcriptome comparison. In both ethylene and firmness column, the first data reported refer to the control sample (Ctrl), while the second to the treated sample (1-MCP).

Stage	DAFB (days)	Ethylene ($\mu l\ kg^{-1}\ h^{-1}$)	Firmness ($kg \cdot cm^{-2}$)
Green	60	N.D	N.D
Breaker	91	N.D	N.D
Red Ripe	114	N.D	N.D
T1	120	Ctrl:16.11	Ctrl: 7.53
		1-MCP: 0.66	1-MCP: 8.09
T2	123	Ctrl: 52.67	Ctrl: 6.03
		1-MCP: 0.42	1-MCP: 8.28

DAFB: days after full bloom; N.D: data not determined.